HHS Public Access

Author manuscript

Mol Cell Endocrinol. Author manuscript; available in PMC 2018 January 05.

Published in final edited form as:

Mol Cell Endocrinol. 2017 January 5; 439: 213–223. doi:10.1016/j.mce.2016.09.005.

Sox21 deletion in mice causes postnatal growth deficiency without physiological disruption of hypothalamic-pituitary endocrine axes

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Abstract

The hypothalamic-pituitary axes are the coordinating centers for multiple endocrine gland functions and physiological processes. Defects in the hypothalamus or pituitary gland can cause reduced growth and severe short stature, affecting approximately 1 in 4000 children, and a large percentage of cases of pituitary hormone deficiencies do not have an identified genetic cause. SOX21 is a protein that regulates hair, neural, and trophoblast stem cell differentiation. Mice lacking *Sox21* have reduced growth, but the etiology of this growth defect has not been described. We studied the expression of *Sox21* in hypothalamic-pituitary development and examined multiple endocrine axes in these mice. We find no evidence of reduced intrauterine growth, food intake, or physical activity, but there is evidence for increased energy expenditure in mutants. In addition, despite changes in pituitary hormone expression, hypothalamic-pituitary axes appear to be functional. Therefore, *SOX21* variants may be a cause of non-endocrine short stature in humans.

Keywords

SOX21; SoxB1; pituitary; hypothalamus; metabolism

1. Introduction

Children with heights more than 2 standard deviations (-2SD) below the mean are defined as being of clinically short stature, affecting those lower than the 3rd percentile for height. Pituitary growth hormone deficiency (GHD) leads to severe short stature and slow growth rates in children, occurring in approximately 1 in 4000 children (1–4), although up to 95% of cases of severe short stature cannot be attributed to endocrine issues (2). Congenital GHD and a lack of other pituitary hormones (hypopituitarism) is most commonly caused by mutations in the gene *PROP1* (5,6), with mutations in *POU1F1* (7) and other genes,

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including genes affecting hypothalamic development such as *OTX2*, accounting for only a small proportion of cases (8–10), leaving the majority of cases of hypopituitarism without a known cause.

The hypothalamus is an important regulator of the endocrine system, consolidating input from the cortex, autonomic system, end organs, and the environment. Hypothalamic neurons deliver neuropeptide hormone signals to the anterior pituitary gland, which in turn secretes polypeptide hormones that regulate the development and function of multiple target organs. Feedback loops between the hypothalamus, pituitary, and end organs form the different levels of the hypothalamic-pituitary axes. These axes regulate growth, lactation, reproduction, metabolism and stress response, in order to maintain homeostasis and respond to physiological demands.

SOX2 and SOX3 are important transcriptional regulators of the developing ventral diencephalon and hypothalamus (11,12). The development and function of the pituitary gland also involves *Sry*-related HMG-box (SOX) proteins: SOX2 and SOX9 are both important transcription factors in the pituitary (13,14), and SOX2 is the primary marker for the postnatal pituitary stem cell population (15,16). SOX2, together with SOX1 and 3, make up the SoxB1 factors, while SOX21 and SOX14 are SoxB2 members (17,18). SoxB1 proteins usually act as transcriptional activators, while SoxB2 proteins are transcriptional repressors (19–21). SOX21 regulates neuronal cell fate decisions (20,22,23), counterbalancing the transcriptional activity of SOX2 (20). SOX21 also regulates trophoblast stem cell differentiation and placentation (24).

Sox21$^{-/-}$ mice are born normally but display cyclic alopecia because SOX21 plays a role in hair shaft cuticle differentiation in follicles of the skin (25). At the same time, *Sox21*$^{-/-}$ mice were noted to be growth deficient without further physiological or endocrine investigations. We considered the possibility that SOX21 is involved in hypothalamic and/or pituitary development and function, and that *Sox21*$^{-/-}$ mutants may have reduced growth because of GHD or thyroid hormone deficiency. We have investigated the function of the hypothalamic-pituitary endocrine axes in these mice and present evidence for postnatal growth insufficiency of alternate origin.

2. Methods

2.1 Animals

The use of mice for this study was approved by the University of Michigan's Institutional Animal Care and Use Committee and the Unit for Laboratory Animal Medicine. Mice were housed under 12 hr light/12 hr dark cycles, and food and water were provided ad libitum. *Sox21*$^{-/-}$ mice were generated by replacing the coding region of the single exon with an enhanced green fluorescent protein (GFP) cassette and removing the floxed neomycin selection cassette with CAG-cre (25). Mice were maintained on a C57BL/6 background. *Sox21*$^{+/-}$ males were bred to *Sox21*$^{+/-}$ females to generate *Sox21*$^{-/-}$ offspring. Males and females were both studied unless otherwise stated. In instances where *Sox21*$^{-/-}$ male and female mice show the same phenotype, only male mice are shown.

2.2 Histology

Tissues from embryonic day (e) 9.5 to postnatal (4-week-old) mice were dissected and fixed in 4% formaldehyde, processed for embedding, and sectioned at a thickness of 6–12 μm. Hematoxylin and eosin stainings were performed with a standard protocol as previously described (10,26) and examined under a light microscope.

2.3 Immunofluorescence

Immunofluorescence was performed on paraffin sections using a tyramide signal amplification system (PerkinElmer, Waltham, MA) as previously described (10,27). The antibodies used in this study were: goat anti-GFP (Abcam Ab5450) at 1:1000; rabbit anti-rat GH (National Hormone and Peptide Program (NHPP), Torrance, CA) at 1:100; rabbit anti-human POMC (NHPP) at 1:150; goat anti-human SOX2 (Neuromics GT15098) at 1:100; goat anti-human SOX21 (R&D Systems AF3538) at 1:100; rabbit anti-mouse GHRH (gift from Frank Talamantes and Malcolm Low) at 1:1000; rabbit anti-mouse GNRH (ThermoFisher PA1-121) at 1:200.

2.4 *In situ* hybridization

In situ hybridization for *Trh* was performed on frozen brain sections using a 957 bp probe from Allen Brain Atlas probe (RP_050725_03_A12) with a non-radioactive protocol as previously described (10), and examined under a light microscope.

2.5 RNA and cDNA production

Pituitary glands and livers were dissected from 4-week-old $Sox2^{+/-}$ and $Sox21^{-/-}$ mice, and total mRNA was isolated using the RNAqueous 4PCR Isolation kit (Applied Biosystems, Life Technologies, Grand Island, NY) according to manufacturer's protocols. cDNA was produced using the Superscript II system (Invitrogen, Life Technologies) according to the manufacturer's protocols (10,26).

2.6 Quantitative PCR

Quantitative real time-PCR was performed on cDNA generated from pituitary and liver tissues from 4-week-old mice using intron-spanning Taqman Gene Expression Assays (Applied Biosciences) as previously described (26). The Taqman Assays used were: *Gh* mm00433590_g1; *Prl* mm00599949_m1; *Lhb* mm00656868_g1; *Tshb* mm00457190_m1; *Pomc* mm00435874_m1; *Igf1* mm00439559. Reactions were set up using Taqman Universal Master Mix (Applied Biosystems), and run on an Applied Biosystems 7500 Real Time-PCR system. Fold changes were calculated by normalizing to the mRNA expression of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) using the Relative Expression Software Tool (REST) program (28).

2.7 Thyroid hormone measurements

Serum total and free thyroxine (T4) were measured using enzyme immunoassay (EIA) test kits (07BC-1007 for total T4 and 07BC-1008 for free T4, MP Biomedicals, Solon, OH). 4-week-old male mice were sacrificed by carbon dioxide inhalation, and blood was collected by cardiac puncture. Sera were used directly for ELISA without dilution. Briefly, sera and

standard solutions of known concentrations of T4 are pipetted into wells coated with anti-T4 antibody, and incubated with a solution containing horseradish peroxidase-labelled T4 for 60 min. The wells are washed with water, and a tetramethylbenzidine solution is added for 20 min to allow color development. The reaction is terminated with 1N HCl solution, and absorbance is measured at 450 nm on a SpectraMax 190 spectrophotometer (Molecular Devices).

2.8 Metabolic and calorimetric analyses

Oxygen consumption (VO_2), carbon dioxide production (VCO_2), spontaneous motor activity, and food intake were measured using the Comprehensive Laboratory Monitoring System (CLAMS, Columbus Instruments). Mice were individually placed into the sealed chambers with free access to food and water, and the study was carried out for 72 hr under with 12 hr light/12 hr dark cycles. Food intake for each animal was monitored through a precision balance under the chamber. VO_2 and VCO_2 in each chamber were sampled sequentially for 5 seconds every 20 min and the motor activity was recorded every second. Total energy expenditure was calculated using the VO_2, VCO_2, and urinary nitrogen concentration (29).

2.9 Imaging and statistical analyses

Images were captured using a Leica Leitz DMB microscope and Leica DFC310 FX camera, or an Olympus FluoView 500 laser scanning confocal system. Images were analyzed and compiled using Adobe Photoshop CS6. All quantitative data show the mean ± standard error of the mean (SEM), and were analyzed by unpaired Student's t-test. In all cases, * =$p<0.05$; ** =$p<0.01$; *** =$p<0.001$.

3. Results

3.1 *Sox21*$^{-/-}$ mice develop postnatal growth deficiency and increased metabolic rate

Sox21$^{-/-}$ mutant mice are visibly smaller in size compared to both their wild-type and heterozygous *Sox21*$^{+/-}$ littermates by 4 weeks of age, with normally proportioned ears and tails (Figure 1A). There is a larger reduction in weight in male mutants than female mutants (Figure 1B) at 4 weeks of age. However, mutant mice are the same weight as their hetereozygous littermates at birth (P0), indicating that the growth deficiency develops postnatally (Figure 1C). Calorimetric studies indicated that mutant mice have increased energy expenditure (Figure 1D), although their food intake is normal (Figure 1E) and they do not have increased physical activity (Figure 1F). Body composition of 4-month-old male mice suggests that mutants have reduced body fat (*Sox21*$^{+/-}$ 18±2% vs *Sox21*$^{-/-}$ 12±0.5%; p=0.055), and increased lean body mass (*Sox21*$^{+/-}$ 64±2% vs *Sox21*$^{-/-}$ 71±0.5%; *, p=0.024).

3.2 SOX21 is expressed in the developing ventral diencephalon but not the pituitary gland

The *Sox21*$^{-}$ allele consists of a GFP expression cassette replacing the coding sequence of *Sox21* (25), causing GFP labeling of *Sox21*-expressing cells. We used detection of GFP signal as a proxy for SOX21 expression in heterozygous *Sox21*$^{+/-}$ mice because the antibody against SOX21 protein can give unspecific background staining (see Figure 3A). In

heterozygous *Sox21*$^{+/-}$ embryos, strong GFP expression is observed in parts of the developing central nervous system, in particular the developing ventral diencephalon, midbrain, and hindbrain, but no GFP expression is seen in the pituitary primordium or Rathke's pouch (Figure 2A). Immunofluorescence in sections of the embryonic and adult hypothalamus and pituitary gland with an anti-GFP antibody (Figure 2B) shows consistent GFP expression in the periventricular cells of the ventral diencephalon, and in the midbrain and hindbrain. However, no staining is observed in the developing or adult pituitary gland, or the developing infundibulum or posterior lobe of the pituitary gland.

3.3 SOX21 is detected in hypothalamic periventricular cells, but no hypothalamic defects were identified in *Sox21*$^{-/-}$ mice

Since SOX21 is detected in the developing ventral diencephalon as early as e10.5, we examined whether *Sox21*$^{-/-}$ mice show hypothalamic defects in 4-week-old mutants. Hematoxylin and eosin staining is normal in *Sox21*$^{-/-}$ hypothalami, which do not show histological defects. Immunofluorescence with an antibody against SOX21 protein indicates that it is most highly expressed in the adult hypothalamus in periventricular cells lining the third ventricle (Figure 3A). Faint staining in other hypothalamic nuclei appears to be non-specific staining because it is still detectable in the *Sox21*$^{-/-}$ mice, whereas periventricular staining is absent in null mutants as expected. SOX2 immunohistochemical staining is strong in periventricular cells, and staining is indistinguishable in mutants, consistent with no effect on SOX2 expression. Axon terminals of growth hormone-releasing hormone (GHRH) and gonadotropin-releasing hormone (GNRH) neurons were detected in the median eminence by immunohistochemical staining (Figure 3B). Thyrotropin-releasing hormone (*Trh*) neurons in the hypothalamic paraventricular nucleus, which regulate the hypothalamic-pituitary-thyroid (HPT) axis (30), are detected in the mutant mice by *in situ* hybridization. No differences in GHRH, GNRH or TRH neurons were noted between normal and *Sox21* mutants.

3.4 Altered hormone expression levels in *Sox21*$^{-/-}$ pituitary glands without GH-axis dysfunction

Very small changes in hypothalamic neuropeptide output can cause substantive changes in pituitary hormone expression and end organ function. In addition, hypothalamic releasing factors are necessary to establish normal populations of specified anterior pituitary hormone producing cells postnatally. For example, GHRH-knockout mice are dwarfed and have severely hypoplastic pituitary glands because of the reduction in growth hormone (GH) cells (31). Thus, we examined pituitary hormone production in *Sox21* mutants, expecting that any alterations would be secondary to hypothalamic input or end organ feedback, given the lack of evidence for *Sox21* expression in the pituitary gland.

The pituitary glands of mutants are reduced in size (Figure 4A), but retain normal and distinct morphology of the pituitary lobes. Total pituitary RNA content is also reduced in proportion to the reduced size of the gland and the mutant body (Figure 4B). Differentiation of endocrine cell-types, such as GH- and pro-opiomelanocortin (POMC)-expressing cells, occurs normally in mutant pituitary glands, and the population size of various cell types appears normal (Figure 4C). Expression of the pituitary stem cell marker SOX2 is also unaffected.

Quantitative PCR (Q-PCR) of 4-week-old pituitary glands indicates that, although cell specification occurred, most of the anterior pituitary hormone mRNA expression levels are changed in the mutants when normalized to the expression of the housekeeping gene *Gapdh* (Figure 4D). Although *Gh* mRNA levels are unchanged in mutant mice, expression of prolactin (*Prl*) and luteinizing hormone-β subunit (*Lhb*) are reduced in both males and females. *Pomc* and thyroid stimulating hormone-β subunit (*Tshb*) are increased in both mutant males and females. Therefore, although SOX21 expression was not detectable in the pituitary gland itself, the global loss of SOX21 causes alterations in pituitary gene expression. Expression of insulin-like growth factor 1 (*Igf1*) in the liver, which is a downstream target for GH, is also not statistically different in mutant mice (Figure 4E). This suggests that the growth defect of *Sox21*$^{-/-}$ mice is not due to GH-axis dysfunction.

3.5 *Sox21*$^{-/-}$ mutant mice are euthyroid and have normal serum T4 concentrations

The increased *Tshb* expression suggested the possibility of pituitary compensation for poor thyroid hormone production in *Sox21*$^{-/-}$ mice. Thyroid glands from 4-week-old mutants appear to have a lipid infiltrate into the mutant thyroid glands. Aside from this, *Sox21*$^{-/-}$ thyroid glands are histologically normal (Figure 5A), with an appropriate number and size of follicles, and normal height of the follicular epithelium. Analyses of the thyroid hormone thyroxine (T4) in the sera of male mice indicates that both total and free T4 concentrations in male *Sox21*$^{-/-}$ mice are normal (Figure 5B), indicating that the animals are euthyroid. Therefore, the increase in pituitary *Tshb* has elicited the appropriate thyroid response, and is unlikely to be the cause of the mutant growth defect. To determine whether SOX21 is expressed in the thyroid gland, we carried out immunofluorescence for GFP. A few scattered cells in thyroid glands from animals of all genotypes were positive for GFP immunostaining (Figure 5C). This low signal must be non-specific background staining because the same degree of staining is observed in *Sox21*$^{+/+}$ thyroids, which do not contain a GFP gene. As a positive control, GFP immunostaining is highly expressed in essentially all of the columnar epithelial cells that line the adjacent trachea of *Sox21*$^{+/-}$ and *Sox21*$^{-/-}$ mice, but no staining is observed in the tracheal cells of *Sox21*$^{+/+}$ mice (Figure 5D). Thus, there is no evidence for *Sox21* expression in the thyroid gland. Since we observed SOX21 expression in the tracheal epithelium which makes up part of the respiratory system, we analyzed histology of mutant lungs, but alveoli and bronchioles in *Sox21*$^{-/-}$ lungs appear histologically normal (Figure 5E).

3.6 Gonads, adrenal glands, and tibial epiphyseal growth plates of *Sox21*$^{-/-}$ mice are histologically normal

The reduced levels of *Lhb* mRNA and elevated levels of *Pomc* transcripts in pituitaries of 4 wk old *Sox21*$^{-/-}$ mice could indicate defects in other hypothalamic-pituitary hormone axes or pubertal delay associated with reduced body size. We explored these possibilities by examining the histology and function of the target organs in 4-week-old and older mutant mice. The seminiferous tubules in mutant males contain mature spermatozoa, indicating proper spermatogenesis (Figure 6). The ovaries of mutants show multiple stages of follicle development, including pre-antral follicles, antral follicles, and corpora lutea, indicating the mutant mice are ovulating. Both male and female mutants were mated with wild-type mice, and they exhibited normal fertility (data not shown). Pregnant female mutants have

difficulties during parturition and in rearing offspring after birth, possibly because of reduced maternal size.

POMC is the pro-hormone for adrenocorticotropic hormone (ACTH), which stimulates glucocorticoid production in the adrenal glands. However, the structure of both the cortex and medulla of the adrenals appear normal, and the mice do not display pathological symptoms of either adrenal hormone deficiency or excess (Figure 6).

Skeletal defects can cause reduced growth, sometimes without obvious disproportionate bodily structures. To explore this we carried out histology of the tibial epiphyseal growth plates (Figure 6). The coin stack structure of dividing chondrocytes in the proliferative zone and enlarged chondrocytes in the hypertrophic zone were indistinguishable in mutant and wild type mice.

4. Discussion

4.1 Loss of *Sox21* in mice causes postnatal growth deficiency

In epidemiological studies of children with severe short stature in different locations around the world, large proportions of those with clinical short stature do not have GHD (1–4). Idiopathic short stature is a term used to describe patients with clinical short stature that cannot be explained after common causes such as pituitary hormone deficiencies have been excluded (32–34). GH treatments are sometimes administered to patients with idiopathic short stature even if they do not have GHD, and this can result in increased growth rate and only modest increases in adult height (35). Causes of idiopathic short stature may include skeletal dysplasia, poor nutrition or gut absorption, chronic illness, or a combination of multiple traits. The etiology of idiopathic short stature is often difficult to pinpoint.

SOX21 mutations in humans have not been described, although interstitial deletions of the long arm of chromosome 13 in humans, which includes the *SOX21* gene, can cause 13q deletion syndrome (36). This is a complex, variable, multiple congenital anomaly syndrome that can include intellectual disability, growth insufficiency, profound craniofacial defects, cardiac abnormalities and effects on numerous organs including endocrine, digestive and genitourinary systems. The contributions of individual genes within the microdeletion to specific features are still not known. One patient with partial GH deficiency was found to have a chromosomal deletion on 13q encompassing *SOX21*, *FARP1*, and *GPC5* (37), although it was not shown whether the loss of *SOX21* in this patient was responsible for the growth insufficiency phenotype.

Sox21$^{-/-}$ mice are growth deficient at 4 weeks of age, but mutant newborns at P0 are a normal weight, indicating that the growth defect develops postnatally and is not caused by intrauterine growth restriction, which can cause long-lasting growth defects in mice and humans (38,39). Postnatal growth reduction can alternatively be caused by changes in feeding behavior and appetite control. The hypothalamus contains multiple neuroendocrine cell-types that regulate feeding and appetite, such as orexin, ghrelin, neuropeptide Y (NPY), *Agouti*-gene related protein (AgRP), and α-melanocyte stimulating hormone (αMSH) (40,41). We showed that SOX21 is expressed in the developing hypothalamus, and

considered whether hypothalamic feeding circuits are affected by the loss of *Sox21*. However, metabolic studies indicated that food intake is normal in *Sox21*$^{-/-}$ mice, and is therefore not likely responsible for the postnatal growth defect. *Sox21*$^{-/-}$ mice do have increased energy expenditure, but their total physical activity is not increased. This suggests increased energy usage by other bodily mechanisms, such as regulation and maintenance of body temperature, as these mice have cyclic alopecia and lose their fur. At the same time, body composition analysis on older male mice suggests a shift from away from body fat towards increased lean body mass, which is similar to *Sox8*$^{-/-}$ mutant mice, which also show reduced growth (42). *Sox8*$^{-/-}$ mice display progressive adult-onset adipose tissue degeneration (43) and low bone mass (44), which are additional potential causes of growth deficiency in *Sox21*$^{-/-}$ mice, but not included within the scope of this study.

4.2 The growth defects of *Sox21*$^{-/-}$ mutant mice are unlikely to be caused by GH-axis dysfunction

Postnatal growth defects can be caused by GH axis dysfunction, as the GH axis does not regulate growth and body size before birth. *Sox21*-null mutant mice were previously generated by Kiso *et al.* (25), who described the role of SOX21 in the skin and hair follicles, and have also been used to study SOX21 function in hippocampal stem cells (22), and cochlea function and development (45). We considered the possibility of hypothalamic-pituitary defects as the cause of the growth defect. However, our experiments together demonstrate that hypothalamic-pituitary axes dysfunction is unlikely to be the cause of the growth deficiency. GHRH axon terminals in the median eminence are present in the mutant hypothalamus; sufficient GHRH apparently reaches the pituitary gland so that somatotropes differentiate normally and express normal levels of *Gh* mRNA; and subsequently enough GH appears to be secreted to stimulate the liver to elicit a relatively normal *Igf1* response. Therefore, all three levels of the GH-axis appear to be intact, and the loss of SOX21 does not appear to directly affect the GH-axis. Additionally, although the weight reduction in 4-week-old *Sox21*$^{-/-}$ mice is similar to other mouse models of severe growth hormone deficiency, such as *Prop1*- and *Pou1f1*-mutant mice (46,47), *Sox21*$^{-/-}$ mice lack the physical characteristics of GH deficiency. *Prop1*- and *Pou1f1*-mutant mice have a shortened snout, small ears, and a short tail due to developmental delay, none of which are seen in *Sox21*$^{-/-}$ mice. This, together with our examinations of the brain, pituitary and liver, further suggest that the GHRH-GH-IGF1 axis in SOX21-null mice is functioning normally.

4.3 Euthyroidism in *Sox21*$^{-/-}$ mice indicates that hypothalamic-pituitary-thyroid axis dysfunction is not the cause of their growth deficiency

Dysfunction of the HPT axis, leading to hyperthyroidism, can also cause growth deficiencies. TRH neurons in the hypothalamic paraventricular nucleus are the major hypothalamic regulator of the HPT axis (30). *Trh*$^{-/-}$ knockout mice also show growth reduction at 4 weeks of age but grow to normal weights by 8 weeks of age (48), and have increased serum TSHβ and decreased serum T3 and T4 (48,49). Older *Sox21*$^{-/-}$ mice continue to be smaller in size at 8 months of age (~40% reduction in weight), and previous reports indicate that even 3-year-old mice continue to appear visually smaller in size (25). Therefore, the *Sox21*$^{-/-}$ mutants appear phenotypically distinct from the *Trh*$^{-/-}$ knockout mice. However, it is also possible that the changes in the HPT axis may be due to regulation

of TRH secretion and pulsatility from the TRH neurons, which are present in the mutants but be functionally impaired in exocytotic release of TRH or reception of input signals from other neurons in hypothalamus and medulla (30).

We find that pituitary gland expression of *Tshb* mRNA is increased in *Sox21*-mutant mice, but ultimately show that serum concentrations of both free and total T4 in mutant mice are normal, suggesting that the feedback mechanisms of the HPT axis are functional in mutant mice despite the increase in pituitary *Tshb*. Therefore, $Sox21^{-/-}$ mutant mice are euthyroid, and HPT axis dysfunction is unlikely to be the cause of the growth deficiency.

4.4 Alterations in pituitary hormones do not appear to affect end-organ development or function

Pomc transcript in the pituitary glands of $Sox21^{-/-}$ mice is increased, while *Lhb* is reduced. However, histological examination of adrenal glands, testes and ovaries of $Sox21^{-/-}$ mice showed no overall changes to these organs. Excessive adrenal hormone production from any source causes Cushing's syndrome in humans, while excessive ACTH production leads to Cushing's disease. $Sox21^{-/-}$ mice have high *Pomc* mRNA expression, suggesting excess ACTH production as in Cushing's disease. However, the primary symptom of Cushing's disease in mice and humans is weight gain and central obesity (50). On the other hand, $Sox21^{-/-}$ mice have a reduced weight and do not display obesity. Therefore, the modest increase in pituitary *Pomc* expression does not directly lead to physiologically significant adrenal overactivity.

Pituitary expression of *Lhb* in $Sox21^{-/-}$ mice is reduced, although both testes and ovaries develop mature spermatozoa and corpora lutea normally, suggesting functional gonadal development and gametogenesis. Therefore, the reduction in *Lhb* does not lead to hypogonadotropic hypogonadism in $Sox21^{-/-}$ mice, and that hypothalamic-pituitary-gonadal (HPG) axis feedback loops are preserved in these mice.

4.5 Hypothalamic-pituitary axes are functionally maintained in the absence of SOX21

SOX21 may have a role in the generation or function of neuroendocrine neurons during hypothalamus development. We demonstrated by Q-PCR that expression of pituitary hormones are increased (*Pomc*, *Tshb*), decreased (*Lhb*, *Prl*), or unchanged (*Gh*) at 4 wks. However, we also show that the pituitary cells themselves do not express SOX21 at any stage examined. Therefore, the changes in pituitary hormone expression may be due to changes in hypothalamic neuroendocrine populations. We found that axon terminals for GHRH and GNRH neurons are present in the median eminence of mutant mice, so that the reduction in pituitary *Lhb* is not due to an absence of GNRH neurons. However, we cannot rule out an impairment of the secretion and release of GNRH in $Sox21^{-/-}$ neurons, which could account for the low *Lhb* expression. Regardless, any dysfunction of the GNRH neuron themselves ultimately does not cause infertility, indicating that the HPG axis is able to respond to changes at individual levels to maintain gonadal function.

At the same time, the increased expression of *Pomc* and *Tshb* in the mutant pituitary could indicate the increased production or secretion of corticotropin-releasing hormone (CRH) or TRH from their respective neurons. On the other hand, a reduction in CRH and TRH could

also cause negative feedback from the adrenal and thyroid glands on the pituitary to increase *Pomc* and *Tshb* expression. Examination of the adrenal and thyroid glands of mutant mice again indicated that, if hypothalamic CRH or TRH neurons were affected by the loss of SOX21, the hypothalamic-pituitary-adrenal (HPA) and HPT axes are again able to respond appropriately to maintain homeostasis.

4.6 SOX21 is expressed in hypothalamic periventricular cells, including tanycyte neural progenitors

SOX21 has previously been studied in the brain and central nervous system for its role in the differentiation of neurons from progenitors in the developing neural tube (20), neural plate (23), and adult hippocampus (22). We demonstrate here that SOX21 is expressed in the developing ventral diencephalon as early as e10.5, and that its main site of expression in the postnatal hypothalamus is in periventricular cells lining the third ventricle. The hypothalamic periventricular zone is made up of a mix of ependymocytes and tanycytes (51), which also express SOX2, and a subset of tanycytes have been shown to be adult neural progenitor cells (51,52). SOX21 is expressed in virtually all of the periventricular cells, so it is possible that SOX21 regulates hypothalamic stem/progenitor differentiation as it does in the neural tube and hippocampus (20,22,23).

Only a subset of tanycytes lining the third ventricle are thought to be neural progenitor cells (51,52), and α-tanycytes self-renew during adulthood to give rise to tanycytes, astrocytes and low numbers of neurons (51). Hypothalamic progenitors turn over slowly and give rise to modest numbers of nascent cell (51), so that we are unlikely to identify subtle changes to postnatal hypothalamic populations without introducing additional genetic markers into the *Sox21*$^{-/-}$ strain. For example, α-tanycyte neural progenitors were identified by long-term lineage tracing using *GLAST-CreER(T2); R26R* mice, and thus the role of SOX21 expression in periventricular cells could be examined by crossing *GLAST-CreER(T2); R26R* strain onto the *Sox21*$^{-/-}$ strain. However, since SOX21 appears to be expressed in all cells lining the third ventricle, which include non-progenitor cell-types, SOX21 may have additional or alterative roles to stem cell regulation.

Conclusions

We have described here that loss of *Sox21* in mice causes postnatal growth deficiency, and investigated possible roles for SOX21 function in hypothalamic-pituitary axes by examining these axes at multiple levels. We find that there are various alterations to pituitary hormone expression, but observed ultimately that the GH-, HPT, and HPG axes appear to be intact and functional in *Sox21*$^{-/-}$ mice, so that the growth deficiency phenotype of these mutant mice cannot be attributed to dysfunction of hypothalamic-pituitary axes. Therefore, although no *SOX21* mutations have been identified in humans, our physiological data in mice suggest that deleterious *SOX21* variants may cause non-endocrine growth reduction in humans.

Acknowledgments

We thank Michelle Brinkmeier for establishing the *Sox21*$^{-/-}$ mouse colony at University of Michigan, and the Unit for Laboratory Animal Medicine pathology laboratory and Ingrid Bergin for performing mouse necropsies. We also thank the University of Michigan Animal Phenotyping Core, Nathan Qi, and Chuck Burant, for helping to conduct

and interpret the metabolic studies (National Institutes of Health P30 DK089503 and DK020572). This work was funded by the National Institutes of Health (R01 HD30428 SAC).

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Highlights

- This is the first study to explore the reduced growth in *Sox21*-null mice
- SOX21 is expressed in the hypothalamus
- Loss of *Sox21* causes postnatal growth reduction independent of hypothalamic-pituitary axes
- *Sox21*$^{-/-}$ mice have increased energy expenditure but normal physical activity and food intake

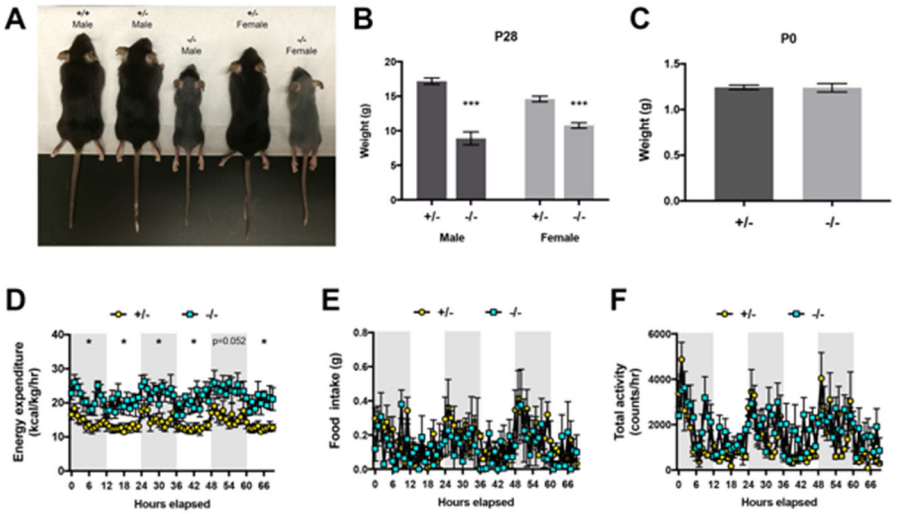

Figure 1. Decreased body weight and increased metabolic rate in postnatal *Sox21*$^{-/-}$ mice

(A–B) 4-week-old *Sox21*$^{-/-}$ mutant mice are reduced in size and weight in both males and females. **(C)** Mutant mice have normal birth weights. **(D)** *Sox21*$^{-/-}$ mice have increased energy expenditure. **(E)** Food intake is normal in mutant mice. **(F)** Total physical activity is not different between mutants and heterozygous controls.

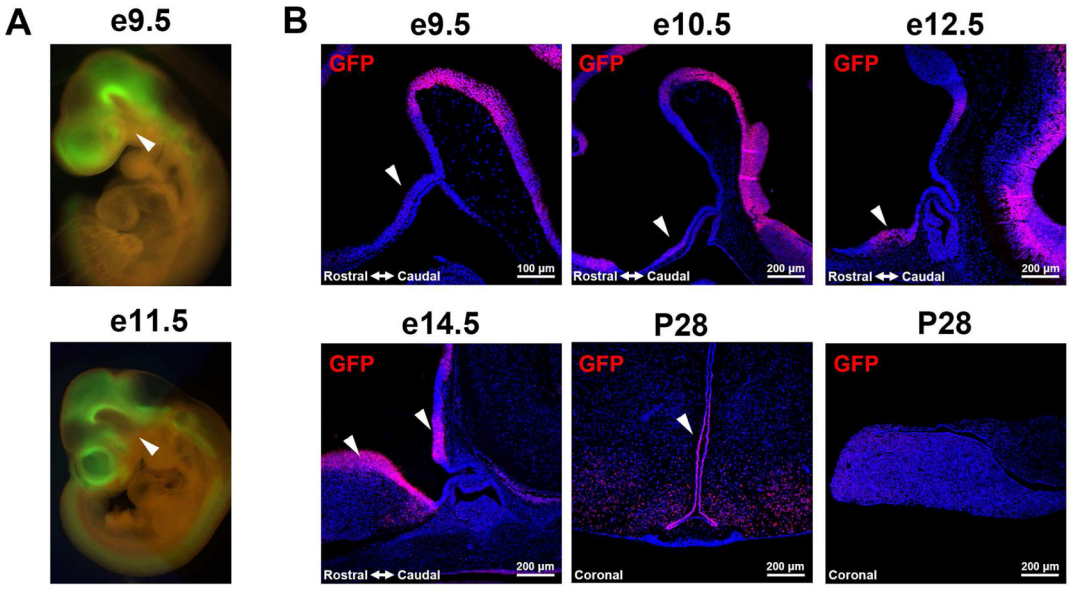

Figure 2. SOX21 expression is expressed in the developing ventral diencephalon

(A) *Sox21*$^{+/-}$ embryos at e9.5 and e11.5 show strong GFP expression in the developing central nervous system but not Rathke's pouch (arrowheads). **(B)** GFP expression is detected in the developing ventral diencephalon e9.5–e14.5, and in the periventricular cells of P28 mice (arrowheads), but is not detected in the developing or P28 pituitary in *Sox21*$^{+/-}$ mice. Arrowheads mark the ventral diencephalon and periventricular cells. *Scale bars as indicated.*

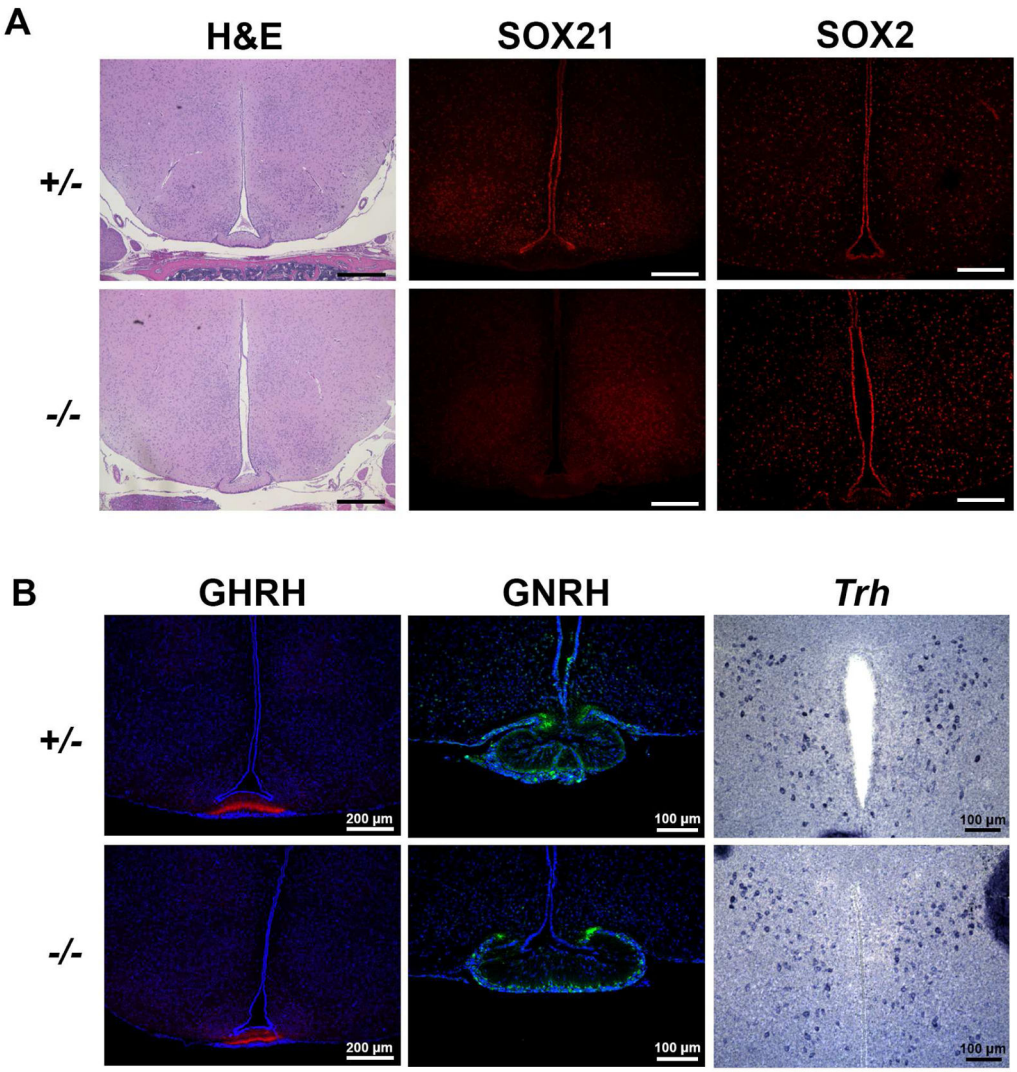

Figure 3. SOX21 is expressed in hypothalamic periventricular cells

(A) Histology of the hypothalamus is normal in mutants. Immunohistochemistry with an anti-SOX21 antibody reveals expression in cells lining the third ventricle in *Sox21*$^{+/-}$ animals, and no signal is detected in *Sox21*$^{-/-}$ mutants. Immunohistochemistry with an anti-SOX2 antibody reveals normal expression in periventricular cells in the mutants. *Scale bar = 200 µm.* **(B)** GHRH, GNRH, and paraventricular *Trh* neurons also appear normal in mutants. *Scale bars as indicated.*

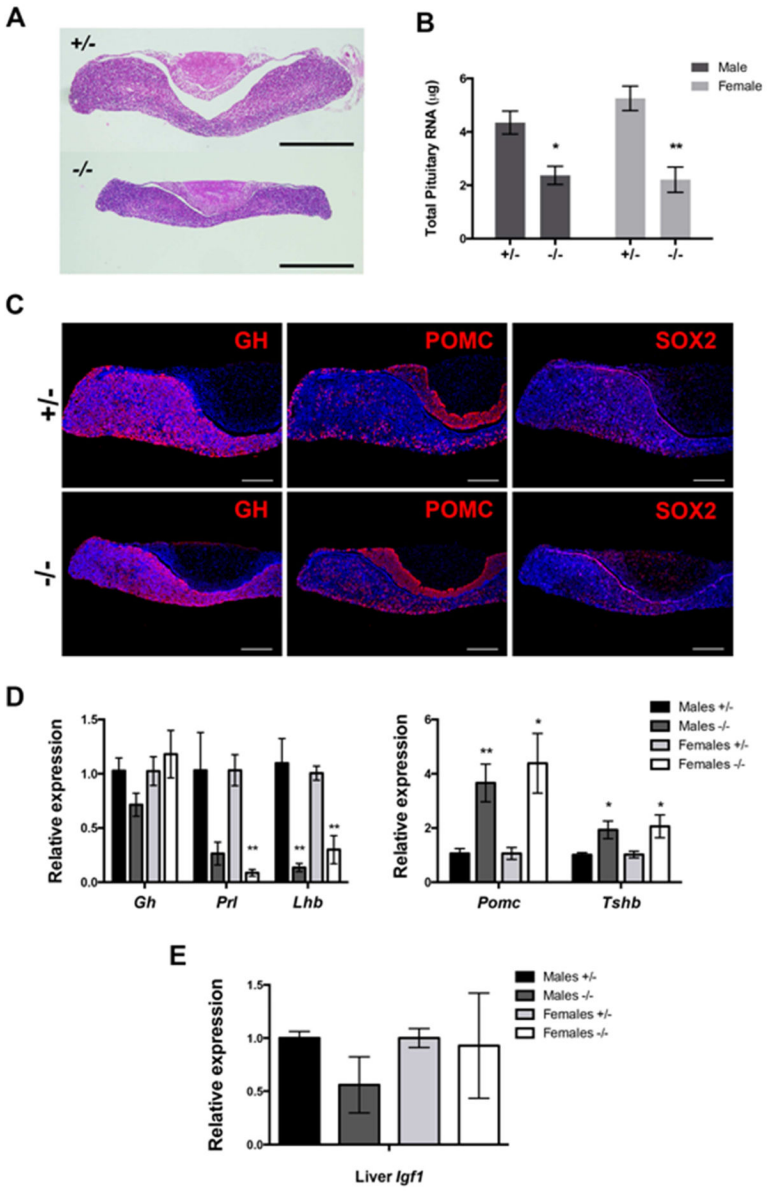

Figure 4. Altered expression of pituitary hormones in *Sox21*$^{-/-}$ mice

(A) Hematoxylin and eosin stained coronal sections from the middle of the pituitary gland reveal reduction in pituitary size in *Sox21* mutants. *Scale bar = 500 µm.* **(B)** Total RNA content is reduced in male and female 4-week-old *Sox21*$^{-/-}$ mutants. **(C)** Specification and population sizes of different endocrine lineages and SOX2 cells are normal in *Sox21*$^{-/-}$ mutants. *Scale bar = 200 µm.* **(D)** Q-PCR reveals normal *Gh* transcript levels in *Sox21*$^{-/-}$ mutants. *Prl* and *Lhb* expression are reduced while *Tshb* and *Pomc* are increased in male and female mutants relative to normal littermates. *n=3–5 for each group.* **(E)** Q-PCR shows that liver *Igf-1* transcript levels are normal in *Sox21*$^{-/-}$ mice. *n=3–5 for each group.*

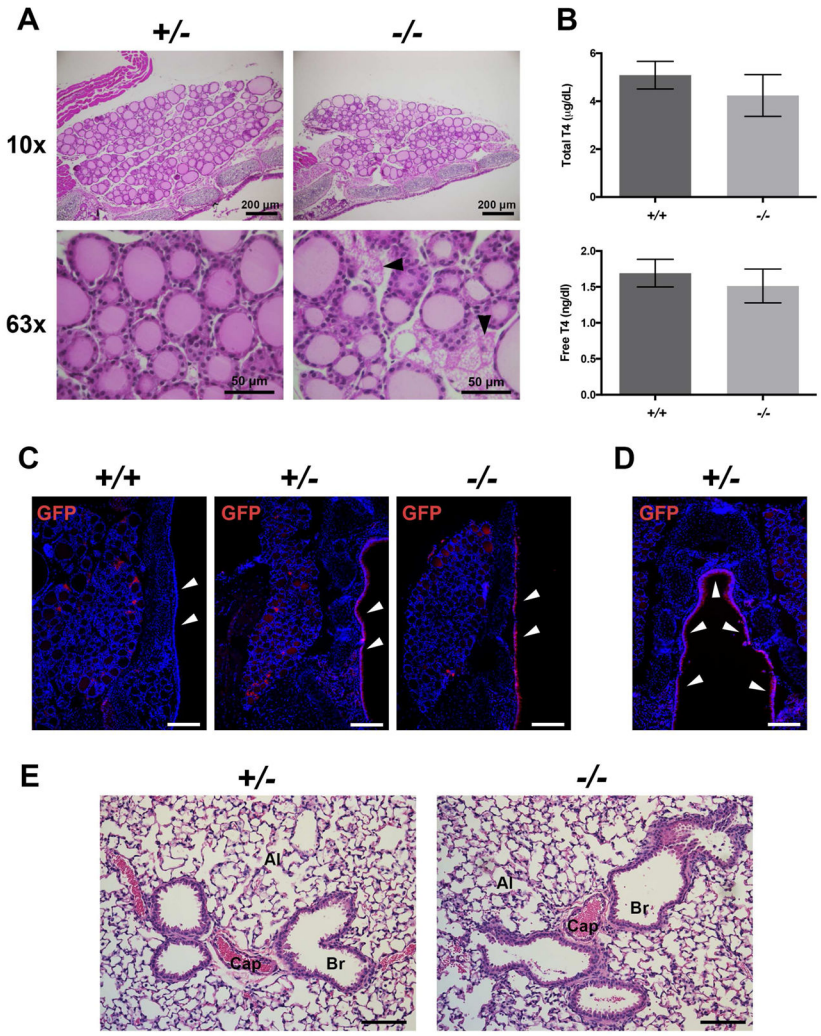

Figure 5. 4-week-old *Sox21*$^{-/-}$ mutants are euthyroid

(A) Hematoxylin and eosin staining of sections from 4-week-old *Sox21*$^{-/-}$ mutant thyroid glands show fatty infiltration between follicles. *Scale bars as indicated.* **(B)** Serum and free T4 concentrations in *Sox21*$^{-/-}$ male mutants are normal. **(C)** Rare, background immunostaining for GFP in the thyroid glands of GFP negative *Sox21*$^{+/+}$ mice is equivalent to staining in GFP positive *Sox21*$^{+/-}$ and *Sox21*$^{-/-}$ mice. Arrowheads indicate tracheal epithelial cells near the thyroid gland, which have bona fide GFP immunostaining in *Sox21*$^{+/-}$ and *Sox21*$^{-/-}$ but not *Sox21*$^{+/+}$ mice. *Scale bar = 200 µm.* **(D)** GFP immunostaining indicates SOX21 expression in the tracheal epithelium. *Scale bar = 200 µm.* **(E)** Hematoxylin and eosin staining of lung sections are indistinguishable between *Sox21*$^{+/+}$ and *Sox21*$^{-/-}$ mutants. *Al=alveoli; Cap=capillary; Br=bronchiole. Scale bar = 50 µm.*

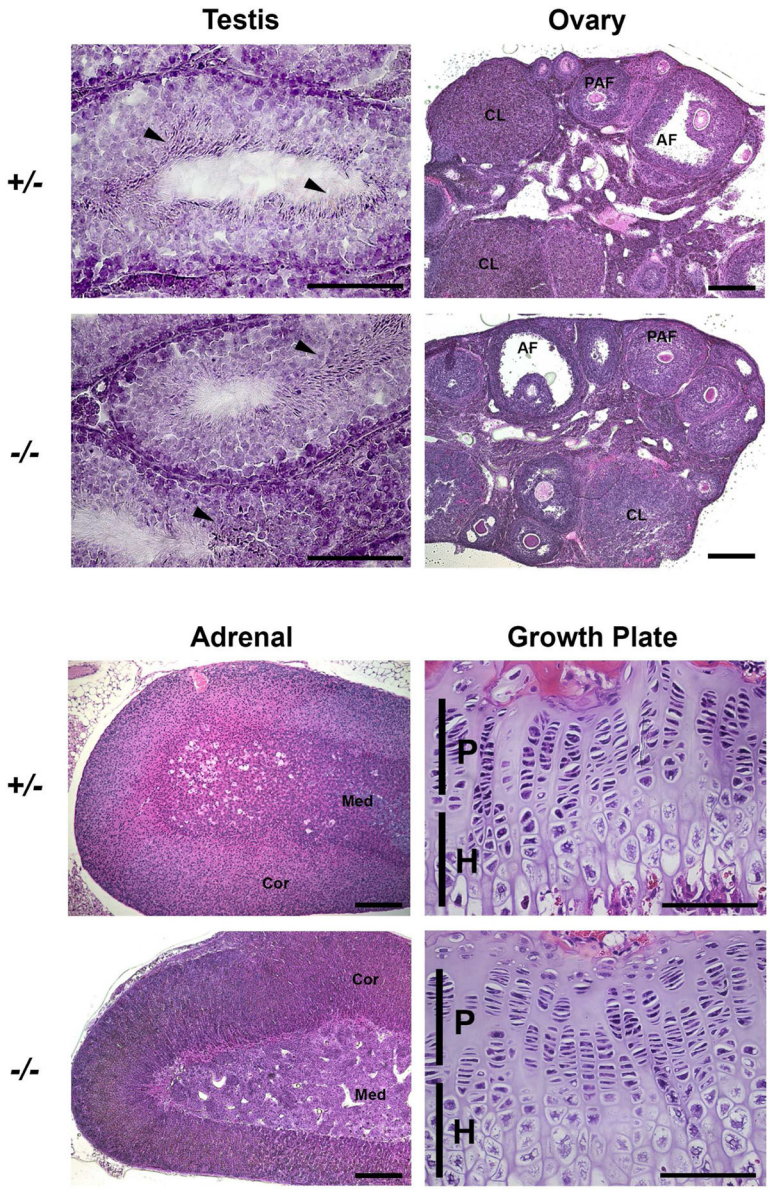

Figure 6. Gonads, adrenal glands and tibial epiphyseal growth plates of *Sox21*$^{-/-}$ male and female mutants are normal

Sections of gonads, adrenal glands and growth plates of mature, adult normal and mutant mice were stained with hematoxylin and eosin. Mature spermatozoa are observed in the mutant testes (arrowheads). *PAF=pre-antral follicle; AF=antral follicle; CL=corpus luteum. Cor=adrenal cortex; Med=adrenal medulla. P=proliferative zone; H=hypertrophic zone. Scale bar = 200 μm.*